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Note

Characterization of haemoglobinous blood substitutes by chromatography and isoelectric focusing with stabilized oxyhaemoglobin as a p*I* marker

T. I. PŘISTOUPIL*, M. KRAMLOVÁ, H. FOŘTOVÁ and V. FRIČOVÁ
Institute of Haematology and Blood Transfusion, Prague (Czechoslovakia)
and
L. KADLECOVÁ
1st Department of Medical Chemistry, Charles University, Prague (Czechoslovakia)
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Chemically modified human and recently also bovine stroma-free haemoglobins (SFH) are prospective oxygen-transporting solutions for infusion therapy and prevention of haemorrhagic shock in emergency situations[1–3] Analytical isoelectric focusing (IEF)[4], chromatofocusing (CF)[5] and fast protein liquid chromatography (FPLC)[6,7] have been found useful methods for the control of heterogeneity and batch reproducibility in the preparation of former human SFH. However, an exact determination of p*I* values of the numerous SFH subfractions in IEF was difficult.

The purpose of this work was to characterize the heterogeneity of recently developed SFH variants and to compare their typical FPLC and IEF "fingerprints". At the same time, we wanted to introduce an improved version of stabilized dry native oxyhaemoglobin (oxyHb) as a suitable coloured marker of the pH gradient in IEF within a pH range of about 6.5–8.

EXPERIMENTAL

SFH was prepared from washed sterile outdated human erythrocytes (up to 42 days of storage in a blood bank) or from fresh bovine erythrocytes by hypoosmotic haemolysis and through removal of stroma as described earlier[4]. SFH was modified in principle by pyridoxal-5-phosphate, sodium borohydride (Fluka, Buchs, Switzerland), glutaraldehyde (Merck, Darmstadt, F.R.G.)[4,8] and binding to human serum albumin (Sevec, Prague, Czechoslovakia)[9]. Dry stabilized oxyHb, batch No. 01-190583, was prepared according to ref. 10. About 10–20 mg of the dry oxyHb standard was rapidly dissolved in 0.2 ml of water and desalted before IEF in a similar manner to the other samples.

Analytical thin-layer IEF was performed as reported earlier[4] on IEF agarose with Pharmalyte 3-10 (Pharmacia, Uppsala, Sweden). Pharmacia calibration kit 3-10 of protein standards for IEF was used to establish a comparative p*I* scale. FPLC was performed as described earlier[6,7] on a Mono Q pre-packed HR5 column (the Pharmacia FPLC system) using for elution a linear gradient of sodiumchloride in a 0.02 *M* Tris buffer (pH 8.0).

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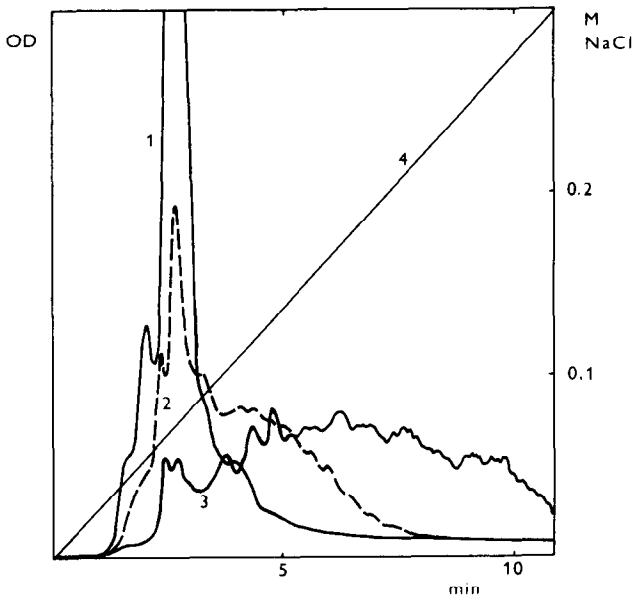

Fig. 1. Fast protein liquid chromatography of haemoglobin samples on HR5 column with Mono Q anion exchanger. 1 = Bovine Hb; 2 = bovine Hb treated with glutaraldehyde; 3 = human Hb modified according to ref. 9; 4 = 0–0.3 *M* sodium chloride gradient in 0.02 *M* Tris buffer (pH 8.0).

RESULTS AND DISCUSSION

FPLC of native and modified bovine Hb and of a new variant of modified human Hb[9] (Fig. 1) shows a marked shift of the Hb subfractions into a complex moiety of more acidic components. However, a part of the fractions around Hb A seems to remain unmodified after chemical treatment. This is in general agreement with former results achieved by FPLC[6,7] and IEF[4] (Fig. 2).

Fig. 2 is a typical IEF run of recent SFH products investigated in our laboratory. All samples show a high degree of heterogeneity characterized by about 10–17 subfractions of very different intensity. Each type of SFH has its individual IEF fingerprint. Spontaneous oxidation to methaemoglobin (metHb) was more rapid in bovine Hb than in human Hb during isolation. This is why the zones B_1B_2 (half-oxidized Hb tetramers) and A^+ (fully oxidized fraction of Hb A) are more intense in bovine than in human Hb. Lyophilization of bovine Hb with sucrose did not change the IEF pattern except for the appearance of a new non-haeme protein zone near p*I* 5.5. Pyridoxalation and glutaraldehyde treatment of bovine Hb leads to the formation of a moiety of acid Hb conjugates and to some fusion of most zones. Part of the neutral and alkaline Hb subfractions (about 20–25%) seems to remain unaltered during modification (as had been observed with human Hb)[4,6,7].

Stabilized human oxyHb is a very useful coloured p*I* marker in IEF and permits a visual check of regular progress of the fractionation procedure during IEF runs. It also helps in deciding the right moment to stop the run when the sharpest zone separation has been achieved. After staining, the oxyHb standard usually forms 12–17 sharp zones which indicate the p*I* gradient in very fine intervals between pH

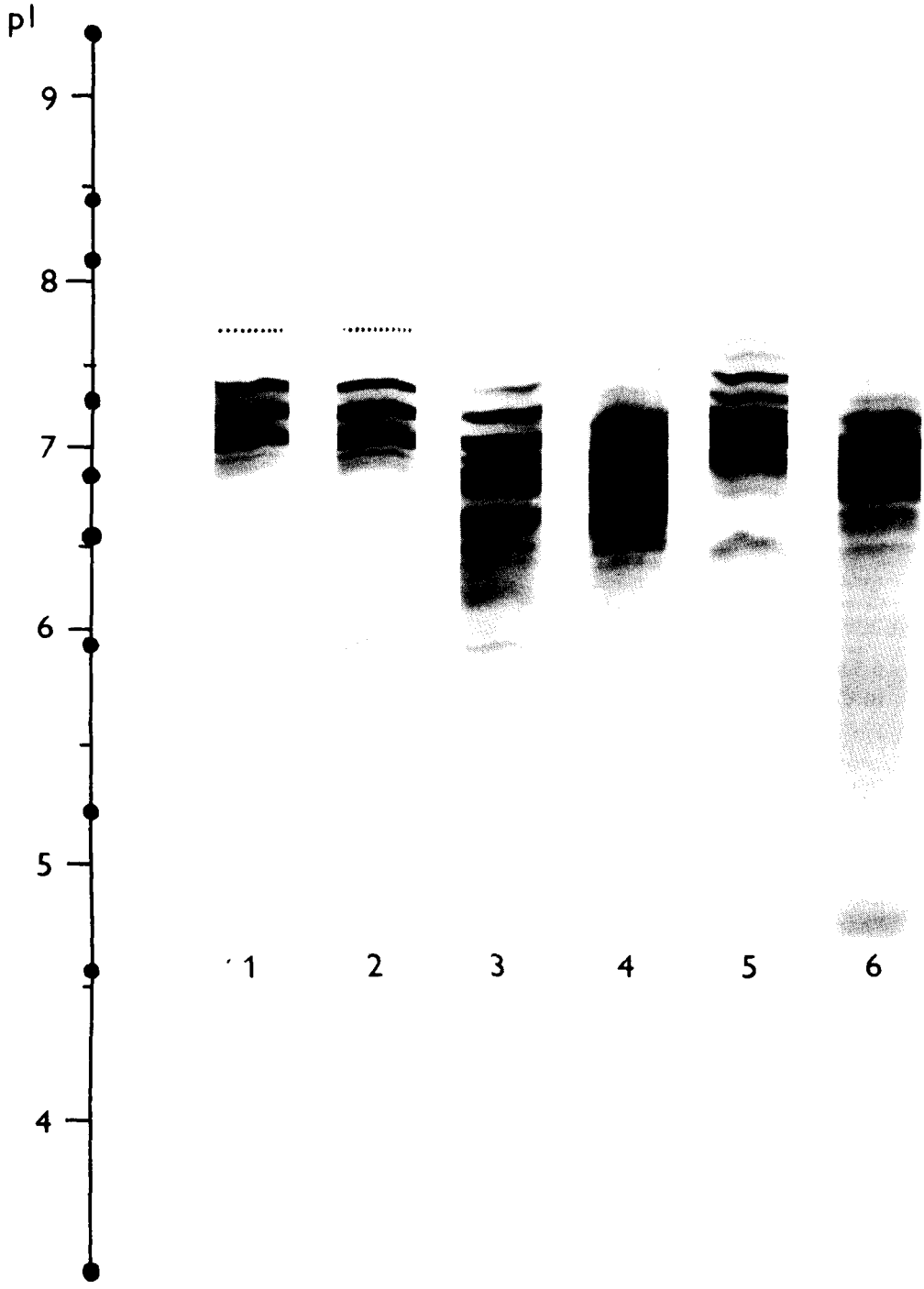

Fig. 2. Isoelectric focusing of haemoglobin samples. 1 = Bovine Hb; 2 = bovine Hb lyophilized with sucrose; 3 = pyridoxalated bovine Hb; 4 = glutaraldehyde-treated sample 3; 5 = human oxyHb (p*I* marker); 6 = human Hb modified according to ref. 9. Pharmalyte 3–10, agarose IEF, stained with Coomassie blue. p*I* scale interpolated from comparative Pharmacia calibration kit 3-10; dark circles indicate the positions of zones.

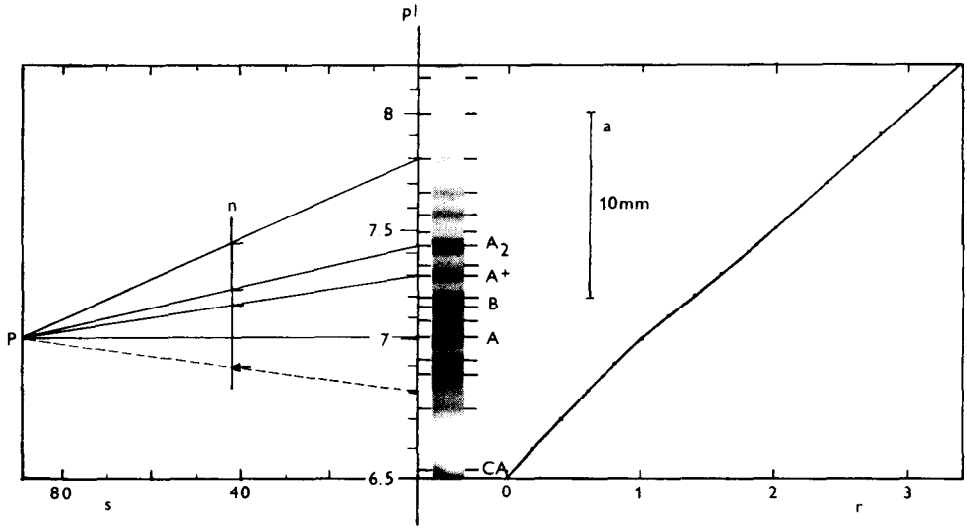

Fig. 3. Details of the oxyHb p*I* marker and nomogram for p*I* determination. Left: nomogram (for its use see text). Full lines correspond to calibrating zones; dashed line corresponds to a sample to be analysed. n = Mutual positions of zones (example); s = auxiliary linear scale; p*I* = scale adjacent to oxyHb standard IEF pattern with indication of the main zones (center). Right: p*I* *versus* distance plot. a = Comparative abscissa to IEF run; r = random linear scale.

6.5 and 8.7. The marked red A, B and A_2 zone and the brown A^+ zone make it easy to discern the remaining zones and to establish a p*I* scale (Figs. 2 and 3) by comparison with the Pharmacia kit and empirical interpolation. The standard deviation (S.D.) in four parallel IEF runs did not exceed 0.02 pH unit within the Hb subfractions. However, the S.D. was 0.15 pH unit with carbonic anhydrase (CA) owing to irregularities in the shape and position of its zone. The p*I* *versus* distance plot shows only a slight deviation from linearity (Fig. 3).

The dry stabilized oxyHb standard remains ready for use for 1–2 years. Its metHb content is usually 5–20% of the total Hb (in contrast to commercial dry "haemoglobin" with about 80% of metHb). The stability against spontaneous oxidation depends on the lyoprotector, temperature, time and humidity[10–14].

The following procedures proved useful in determining precisely the p*I* value of an unknown sample that had been run together with oxyHb during IEF: (1) optical projection and magnification of the transparent IEF pattern on the central section of Fig. 3 to give identical sizes of both calibrating patterns, the p*I* value of the unknown zone is then read on the adjacent p*I* scale (Fig. 3); (2) the positions of the calibrating zones A (p*I* 7.02), A^+ (p*I* 7.29) and A_2 (p*I* 7.44) and the zone of p*I* 7.8, and also that of the unknown sample, are marked together on a paper strip with sharp pencil. The calibration marks are applied on the nomogram in Fig. 3 to coincide with the calibration lines. An auxiliary line (dashed line in Fig. 3) drawn between point P and the unknown zone (arrow) intersects the p*I* scale at the wanted value (e.g., 6.79 in Fig. 3).

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